Serological Specificity of Type E Botulinal Toxin (35708)

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Botulinal toxins and antitoxins are immunologically quite specific as determined by animal neutralization tests (1). Considerable cross-reactivity and heterogeneity, however, have been observed in *in vitro* serological studies with types A and B botulinal toxins (2). Prerequisite to the development of *in vitro* tests for botulinal toxins is an understanding of serological properties. For this reason, type E botulinal toxin was investigated by passive hemagglutination and immunodiffusion. Potentially, more information on the complexity of botulinal toxins can be obtained by antigenic studies.

Materials and Methods. Purified type E botulinal toxin. Toxin purified as described by Kitamura *et al.* (3) was obtained from Dr. G. Sakaguchi, Osaka Prefectural University, Osaka, Japan. The toxin is described in this paper as "purified Sakaguchi."

Partially purified type E botulinal toxins. The Alaska, Beluga, Tenno, and 35396 (obtained from Dr. Sakaguchi) strains of *Clostridium botulinum* type E were grown in sac cultures, and the toxins were partially purified by ammonium sulfate precipitation as previously described (4). Strain 35396 was employed in the production of the purified toxin, and its partially purified toxin here is referred to as "partially purified Sakaguchi." The culture medium for toxin production was prepared as described by Gordon *et al.* (5).

Toxicities. Mouse LD_{50} determinations were performed on trypsin-activated toxins (4).

Red cell couplings. Toxins were coupled to sheep red blood cells (SRBC) with bisdiazotized benzidine (BDB) as previously described (2). For purified E toxin, 25 μg protein/ml and 0.3 ml BDB were used. For partially purified toxins, 165–215 μg protein/ml and 1.0 ml BDB were used.

Antitoxins. Rabbit antitoxin to E toxin was obtained from Dr. Sakaguchi. Antitoxin to type E toxin was also produced in rabbits by repeated injections of type E toxoid obtained from the U.S. Army Biological Laboratories (2). A third antitoxin was obtained from Dr. H. Sugiyama of the University of Wisconsin. It was produced in rabbits that were primed with toxoid and boosted with partially purified toxin (6).

Hemagglutinations (HA). HA with purified and partially purified E toxins coupled to SRBC by BDB were performed as described (4). The volumes and concentrations of the reagents were adjusted for microtitrations.

Hemagglutination inhibitions (HI). Hemagglutination inhibitions were performed as previously described (2) except on the microtitration scale. Nontrypsinized toxins were used.

Immunodiffusions. Ouchterlony gel diffusions were carried out with 2 × 2 in. templates as described by Johnson (7).

Results. The specific toxicities of the purified Sakaguchi type E botulinal toxin and several sources of partially purified E toxins are presented in Table I. The purified toxin had a specific toxicity that ranged from 30 to 550 times greater than that of the partially purified preparations.

The purified toxin and partially purified toxins were used both as SRBC antigens and inhibitors in the HI presented in Table II. As inhibitors, all toxins were used at a concentration of 2000 mouse LD_{50}/ml. Although

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TABLE I. Toxicity of Purified and Partially Purified Type E Botulinal Toxins.

Toxin	Mouse LD_{50}/mg protein
Purified Sakaguchi	5.5×10^8
Partially purified Sakaguchi	1.84×10^5
Alaska	1.78×10^6
Beluga	1.01×10^4
Tenno	1.26×10^4

the degree of inhibition by a given inhibitor varied with the SRBC antigen and antitoxin, certain distinct patterns were observed. Purified Sakaguchi, for example, was inhibitory only when it was also used as the SRBC antigen. Beluga and Tenno, the toxins with the lowest specific activities, were the most effective inhibitors with all SRBC antigens and antitoxins. Partially purified Sakaguchi and Alaska toxins had higher specific activities than Beluga and Tenno but were not as effective as inhibitors, though they exhibited some inhibition with all of the SRBC antigens. Since the concentrations of the inhibitors are based on specific toxicities, the amount of protein used in the inhibitions varied for each toxin. Thus, 2000 LD_{50} of purified Sakaguchi toxin consisted of 0.36 μg protein/ml, whereas the same potency of Beluga toxin consisted of 200 μg protein/ml. Types A and B botulinal toxins from several sources (2) were noninhibitory.

Table III compares the relative inhibitory ability of purified Sakaguchi and partially purified Beluga toxins with each inhibitor used at 100 μg/ml. With Sakaguchi as the SRBC antigen, the purified toxin was the most effective inhibitor. With Beluga as antigen, however, the Beluga toxin was the most effective inhibitor. It is of interest that the purified toxin at this relatively high concentration inhibited HA to the extent of only 8- and 16-fold with the Beluga SRBC antigen. The serological specificity, then, of purified type E botulinal toxin differs from that of

TABLE II. Hemagglutination Inhibitions with Various Type E Botulinal Toxins and Antitoxins.

SRBC antigen[a]	Inhibitors, 2000 LD_{50}/ml	Fold reduction in titer: Sakaguchi antitoxin	Rabbit antitoxin	Sugiyama antitoxin
Purified Sakaguchi	Purified Sakaguchi	32	32	128
	Partially purified Sakaguchi	16–32	32	128
	Alaska	16–32	8	128
	Beluga	1024	1024–2048	$\geqq$ 1024
	Tenno	256	512–1024	$\geqq$ 1024
Partially purified Sakaguchi	Purified Sakaguchi	0	0	0
	Partially purified Sakaguchi	16	16	128
	Alaska	16	16	16
	Beluga	1024	1024	512
	Tenno	256	512	512
Alaska	Purified Sakaguchi	0	0	0
	Partially purified Sakaguchi	$\geqq$ 64	0	32
	Alaska	$\geqq$ 64	8	64
	Beluga	$\geqq$ 64	256	$>$ 128
	Tenno	$\geqq$ 64	256	$>$ 128
Beluga	Purified Sakaguchi	0	0	0
	Partially purified Sakaguchi	8	16	128
	Alaska	16	8	16
	Beluga	256	1024	1024
	Tenno	32	512	1024

[a] HA titers for the SRBC antigens varied from 1:1024 to 1:8192 for the three antitoxins in the absence of inhibitors.

TABLE III. Hemagglutination Inhibition with Purified Sakaguchi and Partially Purified Beluga Type E Botulinal Toxins.

SRBC antigen	Inhibitors, 100 μg/ml	Fold reduction in titer: Sakaguchi antitoxin	Fold reduction in titer: Rabbit antitoxin
Purified Sakaguchi	Purified Sakaguchi	≧256	≧2048
	Beluga	64	1024
Beluga	Purified Sakaguchi	16	8
	Beluga	256	512

crude toxins in HI.

The serological specificity of the E toxins was also examined by the immunodiffusion test (Fig. 1). In general, two bands or more were observed with partially purified preparations with the three antitoxins. Only one band was observed for the purified Sakaguchi toxin that was used at a concentration of 100 μg/ml. The single band for the purified toxin showed identity with a band present in the partially purified preparations.

Discussion. The serological specificity of purified Sakaguchi type E botulinal toxin differs from that of the partially purified toxins, including partially purified Sakaguchi, as indicated by HI and immunodiffusions with several sources of antitoxins.

The difference in serological specificities is strikingly demonstrated by the fact that 5.5 × 10^5 LD_{50}/ml (100 μg/ml) of purified toxin inhibited the Beluga SRBC hemagglutination to the extent of only 8- and 16-fold with rabbit and Sakaguchi antitoxins, whereas inhibition of the homologous systems was in excess of 256- and 2048-fold (Table III). Similarly, 2000 LD_{50}/ml (0.36 μg/ml) of purified toxin was more effective as an inhibitor with the homologous SRBC antigen (Table II). Further, the inhibitory pattern of partially purified Sakaguchi toxin differed from that of purified Sakaguchi toxin.

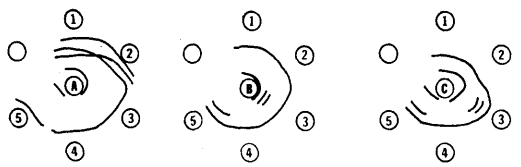

FIG. 1. Diagrammatic representation of gel-diffusion reactions of type E botulinal toxins and antitoxins. A, rabbit antitoxin; B, Sakaguchi antitoxin; C, Sugiyama antitoxin; 1, Alaska toxin; 2, Beluga toxin; 3, Tenno toxin; 4, purified Sakaguchi toxin; 5, partially purified Sakaguchi toxin.

Of interest is the extensive HI by Beluga and Tenno with the purified Sakaguchi toxin SRBC antigen (Table II). All toxins were used at 2000 mouse LD_{50}/ml. If the toxicity determinations truly reflected toxin concentrations, purified Sakaguchi toxin should have been as effective as Beluga and Tenno in the inhibitions involving purified Sakaguchi SRBC antigen. This was not the case and is further evidence that a simple direct relationship does not exist between the toxophore groups on the toxin and the antitoxin. Sugiyama *et al.* (8), for example, demonstrated that trypsin treatment of toxin involved activation of toxin receptors without a concomitant increase in antitoxin combining sites. The data presented here suggest that some type E toxin molecules exist in a nontoxic or less toxic form than others, the nature of which is unknown. Differences, then, in specific toxicities between toxins do not necessarily reflect relative concentrations. Although specific toxicities may be misleading in predicting the relative *in vitro* behavior of E toxins, it would be expected that purified E would be a better inhibitor than Beluga on a weight basis with the purified Sakaguchi SRBC antigen. This, indeed, has been demonstrated to be the case (Table III).

Type E toxins purified by some methods have not shown serological properties distinct from crude preparations (9). It appears, therefore, that type E toxin purified as described for the purified Sakaguchi toxin (3)

is a satisfactory antigen for *in vitro* investigations and immunoassay of type E botulinal toxins.

Summary. A purified preparation of type E botulinal toxin possessed serological properties distinct from those of crude preparations. *In vitro* studies suggested that the specific toxicity of a toxin is not necessarily a true reflection of its relative concentration, and that some type E botulinal toxin molecules exist in a nontoxic or less toxic form than others. Although specific toxicities may be misleading in predicting the relative *in vitro* behavior of E toxins, it would be expected that purified Sakaguchi toxin would be a better inhibitor in HI than partially purified toxins on a weight basis with purified Sakaguchi SRBC antigen. The purified Sakaguchi toxin appears to be suitable for *in vitro* investigations and immunoassays.

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